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LIPOSOME-ENCAPSULATED HEMOGLOBIN FOR EMERGENCY  
RESUSCITATION(U) NAVAL RESEARCH LAB WASHINGTON DC  
M C FARMER ET AL. 01 OCT 84

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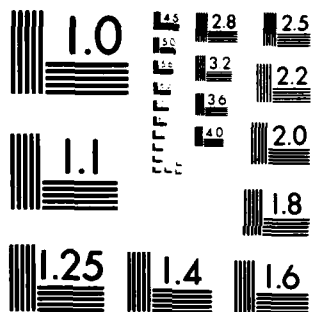
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<p>An oxygen-carrying resuscitation fluid has been developed based upon liposome-encapsulated hemoglobin (LEH). The hemoglobin so encapsulated carries oxygen with unimpaired efficacy. The LEH is stable in the presence of serum and red cells in vitro and displays rheological properties similar to red cells. When tested ex vivo, encapsulation provides some protection from the vasoconstrictive effects seen with stroma-free hemoglobin. In vivo measurements the circulation persistence of LEH in mice exceeds a half lifetime of 6 hours, with animal survival extending beyond two months. An improved lipid formulation substitutes hydrogenated soy lecithin for dimyristoyl lecithin, providing a significant cost reduction and replaces dicetylphosphate with a negatively charged lecithin, to eliminate possible toxic effects. Preliminary data suggest that a major production scale-up can be achieved using commercially available equipment. Objectives for FY85 include: verification of efficacy by total "wash-out" transfusions in rats; improvements in circulation persistence through manipulation of liposome size and composition; determination of the rate of circulation clearance of lipid and hemoglobin; assessment of the effect of LEH on clotting time and immunogenicity; significant production scale-up.</p>				
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**ANNUAL REPORT FISCAL YEAR 1984**

**LIPOSOME-ENCAPSULATED HEMOGLOBIN FOR  
EMERGENCY RESUSCITATION**

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## INTRODUCTION

The purpose of this program is the development of an oxygen-carrying emergency resuscitation fluid based on liposome-encapsulated hemoglobin (LEH).

Liposomes are produced by extrusion of a suspension of lipids in a buffered hemoglobin solution through a graded series of controlled pore-size membranes. Several phospholipid compositions have been prepared and studied for suitability in this application. All are based on phosphatidyl choline (PC) and cholesterol (Chol) with 5 to 10% negatively charged lipid, either dicetyl phosphate (DCP) or dimyristoyl phosphatidylglycerol (DMPG). The hemoglobin is prepared from outdated human blood, carefully separated from erythrocyte membrane fragments. The extrusion procedure produces a highly monodisperse suspension of large nearly unilamellar vesicles (LUV's) with the preselected diameter of 0.2 to 1.0 microns, as determined by photon correlation spectroscopy (PCS). The encapsulated hemoglobin binds oxygen reversibly and cooperatively, with binding parameters comparable to those of the precursor solution. Ions encapsulated with the hemoglobin, such as inorganic and organic phosphates, alter the binding parameters, decreasing the oxygen affinity in the usual manner. Oxidation of the hemoglobin to methemoglobin proceeds very slowly and the LEH binds oxygen reversibly for many months when stored at 4 C. A patent application has been filed describing the preparative procedure.

## OBJECTIVES FOR FISCAL YEAR 1984

Our research objectives this year have been in three areas: 1) Bioengineering, 2) Biophysics, and 3) Physiology. Bioengineering research has been undertaken with the intent of scaling-up the preparative procedure, including exploring new industrial-scale methodologies for liposome manufacture. In addition we have focused on basic problems of biophysics and physiology, including *in vitro* and *in vivo* experiments to assess the interaction of LEH with blood components and whole organ systems. From the physiological standpoint, LEH must be capable of oxygen delivery and circulation persistence sufficient to sustain life until whole blood can be administered. Thus our objective has been *in vivo* testing of the persistence of several lipid mixtures in concert with *in vitro* biophysical experiments aimed at determining the factors which influence the efficiency of solute encapsulation and thus oxygen carrying capacity. The process of solute encapsulation has proven to be a fertile area for basic biophysical research. The solute concentration within extruded liposomes is invariably much less than the solute concentration of the extra-vesicular solution. This finding, corroborated by other researchers, has not yet had a satisfactory explanation. While searching for conditions which maximize solute entrapment for practical reasons, one of our objectives has been the search for a mechanism to account for solute exclusion.

## SUMMARY OF PROGRESS

### Interactions of LEH and RBC's

Incubation of LEH with erythrocytes in serum at 37 C for eight hours results in neither hemolysis nor clotting. Charge repulsion due to the presence of the negatively charged lipid dicetyl phosphate permits prolonged storage without aggregation or fusion. Some aggregation of the LEH in serum due to serum free  $\text{Ca}^{++}$  was expected, and does in fact occur. However, the aggregates are easily dispersed by gentle agitation (Fig. 1). EDTA reverses the aggregation, verifying that fusion does not occur (Farmer & Gaber, 1984).

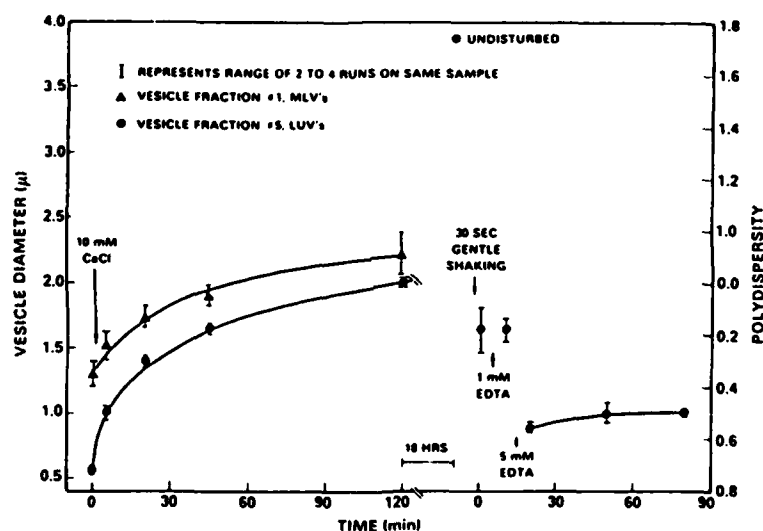


Figure 1.  $\text{Ca}^{++}$  induced change in particle size as measured by PCS. The vesicles contain hemoglobin in phosphate buffered saline, pH 7.4, and are suspended in isotonic NaCl at 22 C.

### Viscosity

The viscosity of LEH suspensions and RBC suspensions decreases as the shear rate increases, with the two materials exhibiting similar patterns of non-Newtonian behavior (Fig. 2). The viscosity of the LEH is very similar to that of whole blood, as measured by cone and plate viscometry. The low shear forces at which the curves change slope markedly indicate that dispersal of aggregates is occurring, and these shear forces are well below those encountered in the microcirculation.

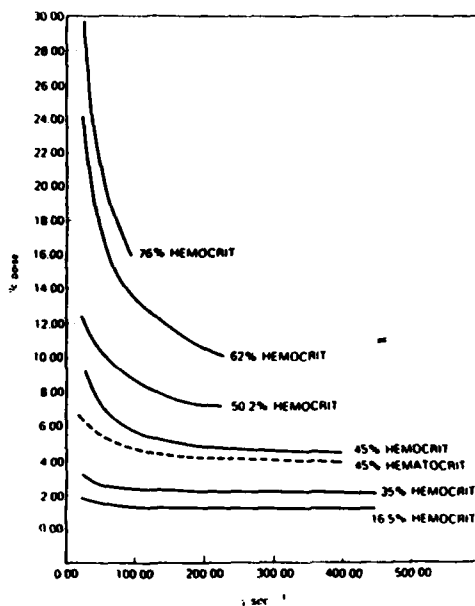


Figure 2. The viscosity of LEH suspensions and RBC suspensions decreases as the shear rate increases, exhibiting non-Newtonian behavior.

#### Isolated rabbit heart

Stroma-free hemoglobin solutions have been found to cause marked vasoconstriction in the isolated rabbit heart. Bradycardia as well as renal complications were reported when such solutions were administered to volunteers (Savitsky, 1978). We have determined that liposome-encapsulation of the hemoglobin provides some protection from the vasoconstrictive effect in the isolated rabbit heart (in conjunction with Drs. Valeri, Dennis and Vogel at the Naval Blood Research Laboratory in Boston).

#### Circulation Half-life

We have developed an accurate simple method for measuring circulation half-life using small aliquots of blood drawn into a hematocrit tube. The LEH is less dense than RBC's or the "buffy coat." Consequently the LEH component in the sample sediments last as a discrete "hemocrit" layer. Disappearance of a bolus of LEH can be measured visually immediately, without the need of radioactive isotopes. A 0.8 ml dose of LEH prepared with dimyristoyl phosphatidylcholine (DMPC):Chol:DCP persisted in the circulation of mice with a half-time of 5.5 hours. All eight mice were maintained for two months subsequently with no apparent toxicity.

Figure 1 is a line graph showing the percentage of initial dose remaining over time (hours) for various doses of 1,2,3,4-tetrahydro-6-methyl-6,6-dimethyl-5H-benzothiazepine-5-oxide. The y-axis is labeled '% OF INITIAL DOSE' and ranges from 0 to 100. The x-axis is labeled 'TIME (HOURS)' and ranges from 0 to 24. Multiple curves are shown, representing different doses. The highest curve is labeled 'LARGE DOSE' and the lowest curve is labeled 'SMALL DOSE'. All curves show a rapid initial decline followed by a slower, linear decrease.

**PERISTANCE TIME TO 50% OF INITIAL "HEMOCRIT" (HOURS)**

**SMALL DOSE = 0.3-0.5 ml  
3-5% "HEMOCRIT" INITIALLY**

**LARGE DOSE = 0.7-0.8 ml  
7-10% "HEMOCRIT" INITIALLY**

Emulsion	Small Dose (Hours)	Large Dose (Hours)
DMPC:chol:DMPG	~2.0	~4.2
DMPC:chol:DCP	~2.6	~5.8
DSPC:chol:DCP	~3.5	~10.0
HSPC:chol:DCP	~3.5	~11.2

**Figure 4. Dose size and phospholipid dependence of LEH circulation persistence.**

### Effect of Charged Lipids

Dicetyl phosphate (DCP) is now thought to be toxic in large doses (G. Poste, personal communication) therefore the mixtures DMPC:chol (no DCP) were tested as well. Our studies of vesicles had indicated that the negative charge might be unnecessary, since fusion of vesicles does not occur even in the presence of high levels of  $\text{Ca}^{++}$  and aggregation was readily reversible (Farmer & Gaber, 1984). However hemoglobin encapsulation in the absence of DCP was reduced from 45% of the extra-vesicular concentration to only 10%. Juliano et al (1984) have infused liposome-encapsulated amphotericin B to treat patients with systemic fungal infections. Their formulation includes 30% dimyristoyl phosphatidylglycerol (DMPG) and neither they nor Poste report toxicity from this lipid which is a normal component of platelet membranes. Therefore we substituted DMPG for DCP and produced LEH which behaved similarly (Fig. 4).

A further evaluation of the role of charged lipid in solute encapsulation is now underway and we can report the following preliminary results. Predictably, an increase in the percentage of charged lipid results in an increase in the encapsulated solute concentration. Furthermore it is apparent that the presence of salt in the suspension buffer inhibits the effect of the charged lipids. Encapsulating hemoglobin in a low salt phosphate buffer followed by exchange to isotonic PBS results in a significantly increased encapsulation efficiency. We are now able to encapsulate hemoglobin via the standard extrusion method at 80% of the extra-vesicular concentration.

### SCALE UP

Producing sufficient material for exchange transfusion of rats, and/or baboons in the coming months, has required that a new method be developed which could produce large batches of material. Following a recommendation from Dr. Jeannine Majde of ONR, we engaged the summer services of Dr. Richard Beissinger, professor of Chemical Engineering at Illinois Institute of Technology and an expert in shear stress effects on red blood cells (supported by NRL in-house funds).

Having examined the specifications of several of the available methods for manufacturing liposomes, we concluded last June that the candidate most likely to succeed was an instrument made by Microfluidics Corporation. Among the arguments in its favor are that this method does not involve contact between the suspension medium (hemoglobin solution) and organic solvents. Also, the principle is similar to that which we have been using, in that a suspension of multi-lamellar vesicles is extruded through a small orifice under pressure, creating high shear forces which serve to produce much smaller liposomes. According to the specifications of the instrument, a highly uniform distribution of nearly unilamellar liposomes of a controllable size range can be produced. Hemoglobin for the project is being provided by Dr. Lakshman Sehgal of the Michael Reese Hospital also in Chicago. We at NRL have provided the lipids, determined the experimental conditions, set the parameters

which we considered would constitute success, and have performed most of the analyses of particle size, hemoglobin integrity and encapsulation efficiency.

The requirements for the liposome-encapsulated hemoglobin (LEH) produced from a scaled-up procedure are as follows:

1. The viscosity of the infusable LEH suspension must be not significantly greater than that of whole blood.
2. The oxygen carrying capacity of the Infusate must be a minimum of 4 vol %.
3. The methemoglobin should be maintained below 10%, with negligible increase with storage at 4 C.
4. The average size of the vesicles should be within the range of 0.2 to 0.6 microns, with a reasonably tight size distribution and no particles above 1 micron.
5. The vesicles must be stable in suspension, neither leaky to hemoglobin nor tending to aggregate significantly.

We have examined in detail 3 experimental conditions with the Microfluidizer. With this instrument it is possible to alter the dimensions of the extrusion microchannels, and the pressure driving the pneumatic pump, thus permitting several ranges of shear forces. Initial trials produced vesicles averaging 0.15 to 0.3 microns. Methemoglobin values were somewhat higher than desirable. We are presently characterizing vesicles made under different experimental conditions, and anticipate that the iterative process will be successful. The 45% encapsulation efficiency is similar to that of our standard method. We will be changing the protocol to the buffer and charged lipid formulations mentioned above to verify that the newly determined techniques for increased encapsulation efficiency are effective in the Microfluidizer.

#### OBJECTIVES FOR FISCAL YEAR 1985

##### Verification of efficacy

After establishing a satisfactory protocol for scaled-up LEH production using the Microfluidizer, our next objective, in experiments to be performed in conjunction with Drs. Sehgal, Rosen and Moss at the Michael Reese Hospital in Chicago, is to verify that the LEH can sustain life in an animal in the virtual absence of erythrocytes. The protocol will involve a "wash-out" transfusion to reduce the hematocrit of the animal to 5% or less. We estimate that approximately 50ml of LEH at a hemocrit of 30% will be sufficient to transfuse a rat. Control animals will be injected with a 30% suspension of hemoglobin-free liposomes in the same isotonic buffered saline solution with added Dextran to preserve the colloidal oncotic pressure. These experiments are scheduled for October, 1984.

### Improving circulation persistence

Our preliminary results with HSPC:Chol:DCP indicate that we have doubled the half-life of our original formulation, and that the half-life is a function of the size of the dose. A complete study of the circulation persistence of this new formulation, as produced by the Microfluidizer, is obviously necessary. The influence of negatively-charged lipids on circulation persistence will also be examined.

### Clearance

The extent of hemoglobin release into the serum will be quantitatively determined spectrophotometrically, and the source of any serum hemoglobin will be determined post-transfusionally by cellulose acetate electrophoresis. Uptake by the reticuloendothelial system (RES) appears to occur with very little loss of hemoglobin into the serum. Using special metabolic cages, urine from the treated mice will be collected and the hemoglobin renal clearance measured. As soon as it is feasible, we will prepare labelled LEH and will determine the pattern of tissue deposition and persistence of the infused hemoglobin and lipids in mice.

### Clotting time and immunogenicity

Clotting factors are known to adsorb onto the surface of liposomes. We will determine the effect of infused LEH on clotting time using the Russell's Viper Venom assay. It is also well established that liposomes can be adjuvants for antigens. Human hemoglobin is not a strong antigen in humans naturally, but if it is denatured on the surface of a liposome its antigenic activity may be enhanced. While a single transfusion of LEH would not be expected to precipitate an immune response, it is essential to know if subsequent transfusions would.

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